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Preview

Old Molecules, New Biochemistry

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The study by Dulcey and colleagues in this issue of *Chemistry & Biology* changes our perception of the pathway of 2-alkyl-4-hydroxyquinoline biosynthesis by the opportunistic pathogen *Pseudomonas aeruginosa* and suggests that the biosynthetic protein complex PqsBC is a potential antibacterial target.

Pseudomonas aeruginosa, a major opportunistic pathogen, produces a wide array of bioactive secondary metabolites. Among these are more than 50 4-hydroxy-2-alkylquinolines (HAQs), which differ in the presence or absence of a hydroxyl substituent at the 3-position, in the length of the 2-alkyl or -alkenyl side chain, and in the presence of an *N*-oxide group in place of the quinoline nitrogen (Lépine et al., 2004). 2-heptyl-3,4-dihydroxyquinoline (2-heptyl-3-hydroxy-4(1*H*)-quinolone), termed the "*Pseudomonas* quinolone signal" (PQS), as well as its biosynthetic precursor 2-heptyl-4-hydroxyquinoline (HHQ) act as signaling molecules in quorum sensing, a cell-to-cell communication mechanism that enables bacteria to synchronize their behavior in a growth- and cell-density-dependent manner (reviewed, e.g., by Heeb et al., 2011). Because PQS signaling significantly contributes to the regulation of virulence gene expression in *P. aeruginosa*, the HAQ quorum sensing system is an attractive antibacterial target. Therefore, understanding the HAQ biosynthetic pathway, which has been unraveled in the study by Dulcey et al. (2013) in this issue of *Chemistry & Biology*, not only advances our knowledge of microbial metabolism and biochemistry, but also contributes to the identification of potential targets to interfere with quorum sensing and thus to control virulence.

Whereas the significance of PQS and HHQ as signaling molecules was recognized around the turn of the 21st century, HAQs were originally identified as antibiotic substances. Studies on antibacterial agents released by *P. aeruginosa* actually date back to the late 19th century. They were probably inspired by the observation of Louis Pasteur that animals injected with a mixture of *Bacillus anthracis* and certain other bacteria failed to develop anthrax. Emmerich and Löw (1899) used concentrated cell-free culture fluid of *Bacillus pyocyaneus* (*P. aeruginosa*) to prevent anthrax in infected rabbits. They thought that the active agent released by *B. pyocyaneus* was an enzyme and thus called it pyocyanase. However, isolation of the antibacterial substance by Hays et al. (1945) revealed that it consisted of several structurally related organic compounds, which—"*for historical reasons*"—were named Pyo I–IV. A few years later, the structure of three Pyo compounds was verified by synthesis. A hypothesis on the biosynthetic pathway was first put forward in 1956 by Cornforth and James, who pointed out that "*the structure of the known Pyo compounds (…) suggests a plausible mode of biosynthesis. Condensation of anthranilic acid with a β-keto fatty acid intermediate (…) could lead, with loss of carbon dioxide and water, directly to a Pyo compound*" (Cornforth and James, 1956). To test this hypothesis versus the alternative assumptions that biosynthesis might proceed via kynurenic acid or via 2,4-dihydroxyquinoline (DHQ), Ritter and Luckner (1971) fed isotope-labeled potential precursors to *P. aeruginosa*. Analysis of HAQ degradation products, obtained by ozonization, permitted localization of the labeled atoms as illustrated in Figure 1A. Ritter and Luckner (1971) as well as Bredenbruch et al. (2005) observed that β-keto fatty acids can be used for HAQ biosynthesis; however, the fate of the carbons, especially those at positions 1 through 3, was not analyzed. Considering known enzymatic reactions of the primary metabolism, Ritter and Luckner proposed a "*likely pathway*" of HAQ biosynthesis from anthraniloyl-coenzyme A and β-ketoacyl-CoA.

This long-standing hypothesis, which, due to its alluring plausibility, has been readily adopted, is challenged in the new study by Dulcey et al. (2013). One of their key experiments involved the feeding of labeled decanoic acid to *P. aeruginosa*, which, according to the "old" hypothesis, should undergo β-oxidation to β-ketodecanoic acid, followed by condensation with activated anthranilic acid and loss of the C1 carbon of the β-ketodecanoic acid as CO_2. While the five deuterium atoms of [9,9,10,10,10-$^2H_5$]decanoic acid were incorporated into HHQ, labeling of HHQ with one ^{13}C from [1,2-$^{13}C_2$]decanoate was not observed (Figure 1B).

HAQs derive from a common biosynthetic pathway, which requires the PqsABCD proteins. Hydroxylation at C-3 of HAQ is catalyzed by PqsH, and synthesis of HAQ-*N*-oxides involves the PqsL protein besides PqsABCD. Whereas PqsA and PqsD were characterized as anthranilate CoA ligase and condensing enzyme, respectively (Figure 1A), the roles of PqsB and PqsC remained unclear. To find out whether formation of the quinoline ring occurs stepwise, involving a yet unidentified intermediate, Dulcey et al. (2013) took a classical microbiological approach and performed elaborate cross-feeding experiments using sterilized supernatants of a series of *P. aeruginosa* mutants defective in individual *pqs* genes. The culture supernatant of *pqsB*$^-$ or *pqsC*$^-$ strains when fed to a *pqsA*$^-$ or *pqsD*$^-$ mutant resulted in HAQ production by the latter mutants, so the *pqsB*$^-$ and *pqsC*$^-$ mutants must produce an intermediate that, in the pathway, is downstream of the PqsA- and PqsD-catalyzed reactions. The authors managed to isolate and identify the new intermediate, which is quite unstable, as 2-aminobenzoylacetate (2-ABA). Chemical synthesis

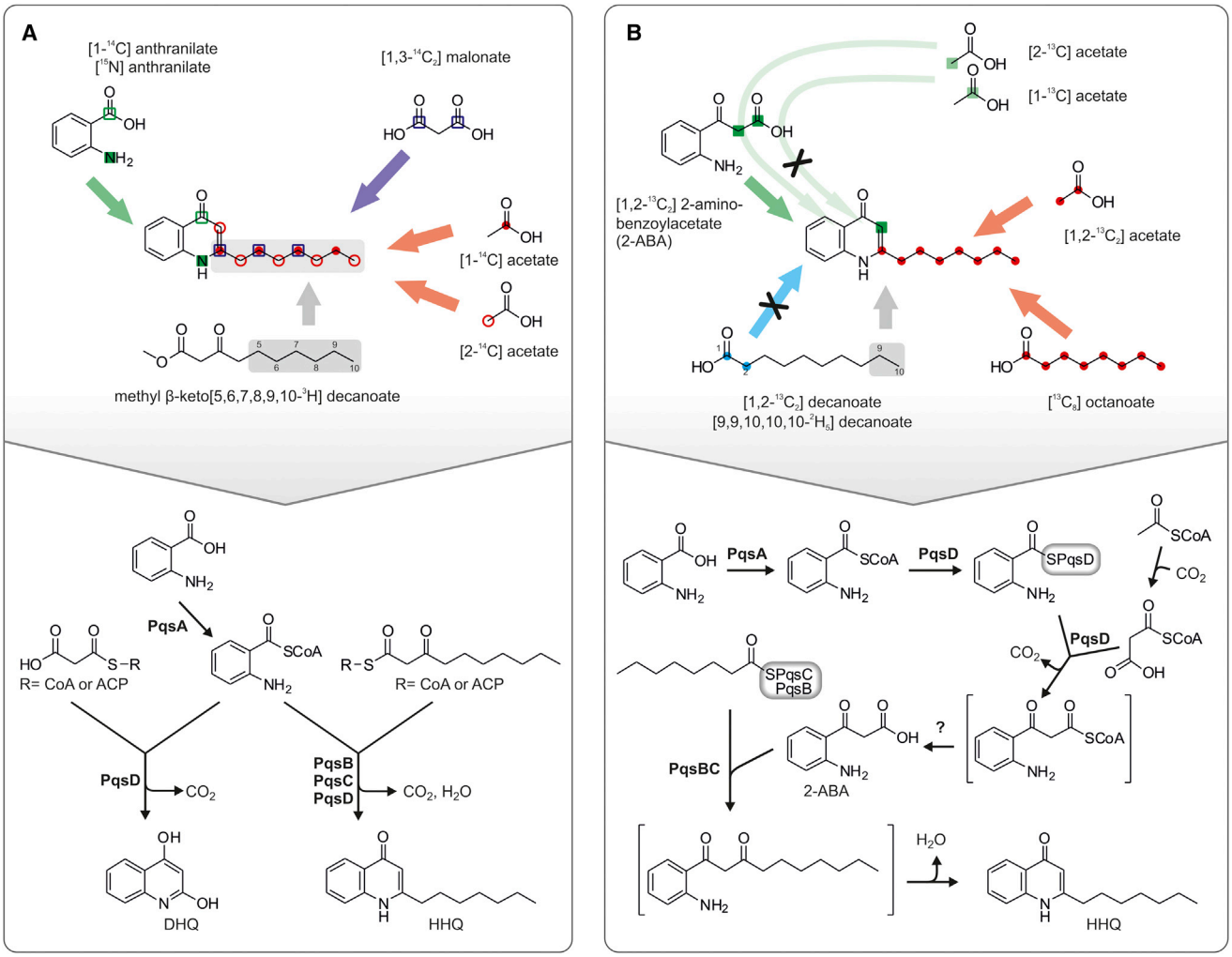

Figure 1. The Long-Standing "Old" Hypothesis on the Biosynthesis of HHQ versus the New Pathway Proposed by Dulcey et al. (2013)
(A and B) The upper parts in (A) and (B) illustrate isotope labeling experiments reported by Ritter and Luckner (1971) and Dulcey et al. (2013), respectively. The lower part of (A) summarizes the "old" hypothetical pathway and indicates the roles of PqsABCD in HAQ biosynthesis (reviewed, e.g., by Heeb et al., 2011); the lower part of (B) shows the pathway of HHQ synthesis as supported by the data of Dulcey et al. (2013). 2-ABA, 2-aminobenzoylacetate; ACP, acyl carrier protein; CoA, coenzyme A; DHQ, 2,4-dihydroxyquinoline; HHQ, 2-heptyl-4-hydroxyquinoline (2-heptyl-4(1*H*)-quinolone).

and isotope incorporation experiments (see Figure 1B) verified its activity as pathway intermediate. When [2-^{13}C] acetate was a precursor of in vivo biosynthesis of 2-ABA, the HHQ formed from the intermediate carried the ^{13}C label. This was not the case if [1-^{13}C]acetate was fed to the 2-ABA producer. Experiments with cell extracts suggested that malonyl-CoA (rather than acetyl-CoA) is the precursor for condensation with activated anthranilic acid. Feeding 2-ABA-containing culture supernatant together with fully ^{13}C-labeled acetate or octanoate to *P. aeruginosa* PA14 (*pqsA*$^{-}$ *pqsH*$^{-}$) revealed that the heptyl side chain together with C2 of the ring can derive directly from octanoate or from de novo fatty acid biosynthesis (Figure 1B). The authors, moreover, performed biochemical studies in order to analyze the functions of PqsB and PqsC. They observed that coexpression of PqsC and PqsB is required to produce soluble protein, which purifies as a stable PqsBC complex. PqsC loading with an octanoyl moiety was shown by peptide mass spectrometry, and PqsBC was proven to be sufficient to catalyze the formation of HHQ from octanoyl-CoA and 2-ABA.

The systematic study of Dulcey et al. (2013) changes our perception of HAQ synthesis and draws attention to the PqsBC complex as potential drug target. However, some questions still remain to be explored. Considering that it catalyzes the condensation of anthraniloyl-CoA with β-ketodecanoic acid in vitro, albeit with a very poor catalytic efficiency (Steinbach et al., 2013), how promiscuous is PqsD? Moreover, a recent report on the interception of octanoyl-CoA by a specific 3-oxoacyl-ACP synthase, which forms β-keto decanoyl-ACP for shunting it into fatty acid biosynthesis (Yuan et al., 2012), poses the question of whether regulatory mechanisms govern the in vivo distribution of octanoyl-CoA. In the new pathway (Figure 1B), is 2-ABA indeed the direct substrate of PqsBC, and if so, which enzyme catalyzes the release of the

acid from a thioester intermediate? Is the formation of 2,4-DHQ and 2-aminoacetophenone by *P. aeruginosa* just an "accident" due to the strong tendency of (activated) 2-ABA to decompose? What is the exact role of PqsB, which belongs to the same protein family as PqsD and PqsC, but lacks the conserved amino acid residues presumed to be essential for the catalytic activity of condensing enzymes? And which of the pathway intermediates is the physiological substrate of PqsL that is required for the synthesis of HAQ-*N*-oxides? Things certainly remain exciting.

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